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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR §1.53(c).

INVENTOR(S)				
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Additional inventors are being named on the <u>0</u> separately numbered sheets attached hereto				
TITLE OF THE INVENTION (500 characters max)				
ENDOPLASMIC RETICULUM (ER) STRESS				
CORRESPONDENCE ADDRESS				
Direct all correspondence to:				
[X] Customer Number: 26161				
OR				
[] Firm or Individual Name				
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Respectfully submitted,

Signature 

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ENDOPLASMIC RETICULUM (ER) STRESS

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made at least in part with government support under grants no. R01 DK067493-01 and DK32520, awarded by the National Institutes of Health. The government has certain rights in this invention.

5

BACKGROUND

Proteins are required for the body to function properly, as they form the basic building blocks of cells, tissue and organ structures. Protein function typically requires the assumption of proper three-dimensional protein structure, which is determined by the amino acid sequence and a process known as protein folding. Sometimes, protein folding goes awry, and misfolded 10 proteins accumulate in cells, causing or contributing to diseases associated with protein misfolding, including amyloidoses (such as immunoglobulin light chain amyloidosis and Alzheimer's disease), Huntington's disease, Parkinson's disease, adult-onset diabetes mellitus, cirrhosis, emphysema, and the prion encephalopathies, alpha-1-antitrypsin deficiency, haemolytic anemia, familial hypercholesterolaemia, amyotrophic lateral sclerosis (ALS), and 15 cystic fibrosis, as well as numerous others. Conformational diseases can be inherited, usually as dominant traits, or can be induced, as in the case of prions.

Proteins destined for secretion such as insulin and alpha1-antitrypsin are translocated into the endoplasmic reticulum (ER) co-translationally; once there, they undergo highly ordered 20 protein folding and post-translational protein processing. However, in some instances, the sensitive folding environment in the ER can be perturbed by pathophysiological processes such as viral infections, environmental toxins, and mutant protein expression, as well as natural processes such as the large biosynthetic load placed on the ER. When the demand that the load of client proteins makes on the ER exceeds the actual folding capacity of the ER to meet that demand, a condition termed "ER stress" results.

Alpha1-antitrypsin (alpha1-AT) deficiency is an exemplary model of a conformational 25 disease. Alpha1-AT is an abundant serum glycoprotein, secreted by the liver, which normally binds to and inactivates elastase, a protease that degrades elastin and collagen. Elastin and collagen maintain the structure of alveoli, air sacs in the lungs. In alpha1-antitrypsin patients, the deficiency leads to uncontrolled destruction of air sacs in the lungs. This condition is called

emphysema and causes a decrease in respiratory function. Alpha1-AT-deficiency mutations interfere with the folding of alpha1-AT, preventing its secretion from the hepatocyte endoplasmic reticulum (ER). Alpha1-AT deficiency is also the leading cause of inherited liver disease in children, caused by the hepatotoxicity of misfolded alpha1-AT molecules that 5 accumulate in the ER lumen.

Cells respond to the accumulation of misfolded proteins in the ER in several ways, including the “ER overload response” and the “unfolded protein response.” The “ER overload response” induces the nuclear transcription factor NF- κ B, a mediator of the immune response. In patients with cystic fibrosis, expression of mutant CFTR induces NF- κ B expression. NF- 10 kappaB upregulates expression of the inflammatory cytokine IL8. Levels of IL-8 are increased in lungs from patients of cystic fibrosis, and NF- κ B was found to be constitutively active in mice in which the wild-type CFTR gene had been replaced with the F508 mutant, supporting the theory that ER stress contributes to the chronic inflammation that often contributes to the high morbidity in cystic fibrosis.

15 The “unfolded protein response” (UPR), triggered by the presence of misfolded protein in the ER, leads to the activation of the kinase Inositol Requiring 1 (IRE1), inducing transcription of genes encoding chaperones and many components of the secretory pathway, and suppresses protein synthesis through a parallel process. In particular, the presence of unfolded proteins in the ER causes dimerization and trans-autophosphorylation of IRE1 that leads to IRE1 activation. 20 Activated IRE1 splices X-Box Binding Protein-1 (XBP-1) mRNA, leading to synthesis of the active form of transcription factor XBP-1 and upregulation of UPR genes (Nature, 2002 Jan 3; 415(6867):92-6; erratum in: Nature 2002 Nov 14; 420(6912):202). In contrast, prolonged ER stress activates the cell death pathway through IRE1.

SUMMARY

25 The present invention provides novel methods and reagents for quantifying levels of ER stress. In particular, the methods feature the use of Inositol Requiring 1 (IRE1) and XBP-1 as specific markers for ER stress level. As a marker, XBP-1 offers an advantage because ER stress levels can be quantified by monitoring the splicing of XBP-1 mRNA. Exemplary methods are based on PCR. For these methods, only a small tissue sample or a small number of cells are 30 required.

Thus, in one aspect, the invention provides methods of quantifying endoplasmic reticulum stress (ER stress). The methods include detecting an IRE1 activation level in a cell or biological sample, wherein the IRE1 activation level correlates with ER stress, and quantifying the IRE1 activation level, such that ER stress is quantified.

5 In some embodiments, the IRE1 activation level is determined by detecting an XBP-1 splicing level, e.g., by amplifying a XBP-1 mRNA region which includes a splice site, or portion thereof, e.g., to create a DNA complementary to the region of the XBP-1 mRNA, e.g., a double-stranded cDNA PCR product; detecting the size of the amplified mRNA (e.g., the cDNA), wherein the size is indicative of spliced or unspliced mRNA. In some embodiments, the levels 10 of spliced XBP-1 are detected and/or the levels of unspliced XBP-1 are detected. In some embodiments, both the levels of spliced XBP-1 are detected and the levels of unspliced XBP-1 are detected, and the ratio of spliced XBP-1 to unspliced XBP-1 is determined. In some embodiments, the amplified mRNA is subjected to restriction enzyme digestion, e.g., Pst I digestion, to facilitate detection of spliced or unspliced mRNA.

15 In some embodiments, the IRE1 activation level is determined by detecting levels of IRE1 autophosphorylation. In some embodiments, the IRE1 activation level is determined by detecting the percentage or ratio of autophosphorylated to unphosphorylated IRE1.

20 In some embodiments, the ER stress level is quantified in a cell, e.g., a mammalian cell, e.g., a human cell, e.g., a pancreatic beta cell. In some embodiments, the ER stress level is quantified in a cell extract, e.g., an extract from a cell as described herein.

In a second aspect, the invention provides methods of diagnosing an ER stress disease in a subject, e.g., diabetes or Wolfram Syndrome, by quantifying the level of ER stress in a cell or biological sample isolated from the subject according to one of the methods described herein; an increased level of ER stress is indicative of the ER stress disease.

25 In a third aspect, the invention provides methods of monitoring the progression of an ER stress disease, e.g., diabetes, in a subject. The methods include quantifying the level of ER stress in a cell or biological sample isolated from the subject at sequential time points according to one of the methods described herein, wherein a change in the level of ER stress indicates the progress of the ER stress disease.

30 In a fourth aspect, the invention includes methods for evaluating the effect of a test compound on ER stress. The methods include providing a providing an ER stress model system

(e.g., a system comprising a cell expressing WFS1, IRE1 and/or XBP-1, e.g., a cultured cell or animal, e.g., a cell or animal model of an ER stress disease); optionally, increasing levels of ER stress in the system (e.g., in the cells or at least some of the cells of an animal); contacting the system with a test compound; and evaluating the levels of ER stress in the system in the presence 5 and absence of the test compound. In some embodiments levels of ER stress are evaluated by measuring XBP-1 splicing, wherein an increase in XBP-1 splicing indicates an increase in ER stress, and a decrease in XBP-1 splicing indicates a decrease in ER stress. In some embodiments, levels of ER stress are evaluated by detecting levels of IRE1 autophosphorylation, wherein an increase in IRE1 autophosphorylation indicates an increase in ER stress, and a 10 decrease in IRE1 autophosphorylation indicates a decrease in ER stress.

In some embodiments, the system is an animal model of an ER stress disease, e.g., an animal model of diabetes (e.g., type 1 or type 2 diabetes), Alzheimer's disease, Parkinson's disease, Wolfram Syndrome, Cystic Fibrosis, familial hypercholesterolaemia or alpha1 antitrypsin deficiency, or cells derived therefrom. Typically, an ER stress disease can be induced 15 in an otherwise healthy animal or cells by administering a compound known to cause ER dysfunction, e.g., by administering a sublethal dose of thapsigargin, tunicamycin (e.g., 0.25–1 mg/kg tunicamycin), or a proteosome inhibitor, e.g., lactacystin.

In some embodiments, the methods include further selecting those test compounds that substantially reduce ER stress (e.g., as measured by IRE1 autophosphorylation levels or XBP-1 20 splicing levels) as candidate therapeutic compounds for further evaluation.

In a fifth aspect, the invention includes a kit for quantifying ER stress. The kit can include primers for amplifying a region of XBP-1 mRNA which includes a splice site, or portion thereof, and instructions for use. In some embodiments, the kit also includes a suitable control.

The invention further includes antibodies that bind specifically to the autophosphorylated 25 form of IRE1, and do not substantially bind the unphosphorylated form. The antibodies can be polyclonal, monoclonal, or monospecific.

The invention also includes an ER stress signaling pathway assay that includes determining the level of ER stress according to one of the methods described herein.

The term "RNA" or "RNA molecule" or "ribonucleic acid molecule" refers to a polymer 30 of ribonucleotides. The term "DNA" or "DNA molecule" or "deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally (e.g.,

by DNA replication or transcription of DNA, respectively). RNA can be post-transcriptionally modified. DNA and RNA can also be chemically synthesized. DNA and RNA can be single-stranded (*i.e.*, ssRNA and ssDNA, respectively) or multi-stranded (*e.g.*, double stranded, *i.e.*, dsRNA and dsDNA, respectively). “snRNA” or “small nuclear RNA” is single-stranded RNA precursor of mRNA. “mRNA” or “messenger RNA” is single-stranded RNA that specifies the amino acid sequence of one or more polypeptide chains. This information is translated during protein synthesis when ribosomes bind to the mRNA. The term “cDNA” or “complementary DNA” refers to a DNA molecule that has a sequence that is complementary to an mRNA or portion thereof, and can include single or double-stranded molecules, but is typically double-stranded.

The term “endoplasmic reticulum stress” (“ER stress”) refers to an imbalance between the demand that the load of client proteins makes on the ER and the actual folding capacity of the ER to meet that demand. A response that counteracts ER stress has been termed “unfolded protein response” (“UPR”).

The term “ER stress disorder” refers to a disease or disorder (*e.g.*, a human disease or disorder) caused by or contributed to by increased ER stress levels. Exemplary ER stress disorders include diabetes (*e.g.*, type 1 or type 2 diabetes), Alzheimer’s disease, Parkinson’s disease, Cystic Fibrosis, familial hypercholesterolaemia and alpha1 antitrypsin deficiency.

The term “protein conformational disease” (“PCD”) refers to a disease or disorder (*e.g.*, a human disease or disorder) associated with protein misfolding (*e.g.*, caused by or contributed to by protein misfolding). Exemplary protein conformational diseases include, but are not limited to, those diseases listed in Table 1. Other diseases include some rare forms of juvenile diabetes, such as Wolcott-Rallison syndrome and Wolfram syndrome; inflammatory Bowel disease; and cancers originated from secretory cells (*e.g.*, breast cancer and prostate cancer).

Table 1: Exemplary Protein Conformational Diseases

Disease	Protein involved
Alzheimer's disease	amyloid- β
immunoglobulin light chain amyloidosis	immunoglobulin light chain
Parkinson's disease	alpha-synuclein
diabetes mellitus type 2	amylin
amyotrophic lateral sclerosis (ALS)	Superoxide dismutase (SOD)
haemodialysis-related amyloidosis	L2-microglobulin
reactive amyloidosis	amyloid-A
cystic fibrosis	cystic fibrosis transmembrane regulator (CFTR)
sickle cell anemia	hemoglobin
Huntington's disease	huntingtin
Kreutzfeldt-Jakob disease and related disorders (prion encephalopathies)	PrP
familial hypercholesterolaemia	low density lipoprotein (LDL) receptor
Alpha1-antitrypsin deficiency, cirrhosis, emphysema	Alpha1-antitrypsin (alpha1-AT)
systemic and cerebral hereditary amyloidoses	(ten other proteins)

Various methodologies of the instant invention include steps that involve comparing a value, level, feature, characteristic, property, etc. to a "suitable control," referred to interchangeably herein as an "appropriate control." A "suitable control" or "appropriate control" is any control or standard known to one of ordinary skill in the art that is useful for comparison purposes. In one embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to performing a methodology of the invention described herein. In another embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined in a cell or organism, e.g., a control or normal cell or organism, exhibiting, for example, normal traits. In yet another embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A is a schematic diagram of the unspliced and spliced mouse XBP-1 mRNAs. The coding regions are boxed, the bZip domain is shaded gray and the 26-base pair nucleotide region

processed by IRE1 is colored black. The active form of XBP-1 mRNA (cDNA) loses 26 base pairs by IRE1 processing. Spliced form of XBP-1 mRNA encodes larger and active form of XBP-1 protein. The inactive form of XBP-1 cDNA is smaller than the DNA fragment of active form of XBP-1.

5 FIG. 1B is a reproduction of a gel stained with ethidium bromide (EtBr) showing the results of RT-PCR analysis done with a primer set encompassing the splice junction of XBP-1 mRNA; PCR products were resolved on 2.5 % agarose gel to separate spliced (active form) and unspliced XBP-1 mRNAs. Wild-type or IRE1 mutant mouse embryonic fibroblast cells were untreated or treated with Tunicamycin (Tm) or Thapsigargin (Tg). Total RNA was prepared at 10 the indicated times. The spliced (encoding active form of XBP-1) and unspliced (encoding inactive form of XBP-1) cDNA fragments are indicated by the arrows.

15 FIG. 2A is a schematic diagram of the unspliced and spliced murine XBP-1 mRNAs. The coding regions are boxed, the bZip domain is shaded grey and the 26-base pair nucleotide region processed by IRE1 is colored black. The active form of XBP-1 mRNA (cDNA) loses its Pst I site by IRE1 processing. The spliced form of XBP-1 mRNA encodes a larger, active form of XBP-1 protein. Thus, the inactive form of XBP-1 cDNA, when digested with Pst I, produces two DNA fragments that are smaller than the DNA fragment of active form of XBP-1 produces when digested with Pst I.

20 FIG. 2B is a reproduction of a gel stained with ethidium bromide (EtBr) showing Pst I digested XBP-1 cDNA from wild-type or IRE1 mutant cells that were untreated or treated with Tunicamycin (TM) or Thapsigargin (Tg). Total RNA was prepared at the indicated times. The spliced (encoding active form of XBP-1) and unspliced (encoding inactive form of XBP-1) cDNA fragments are indicated by the arrows.

25 FIG. 3 is a reproduction of a gel stained with ethidium bromide (EtBr) showing Pst I digested XBP-1 cDNA from mouse islet cells that were untreated (Control) or treated with 1 mM of dithiothreitol (DTT) for 4 hours. The spliced (encoding active form of XBP-1) and unspliced (encoding inactive form of XBP-1) cDNA fragments are indicated by the arrows.

30 FIG. 4 is a reproduction of a gel stained with ethidium bromide (EtBr) showing XBP-1 splicing in MIN-6 cells expressing insulin-2 gene with Akita mutation. Pst I digested XBP-1 cDNA from MIN6 cells untransfected (Control), transfected with wild-type Insulin 2 expression vector (Ins2 WT) or with insulin-2 containing Akita mutation expression vector (Ins2 Akita).

FIG. 5 is text illustrating the mRNA (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences for the spliced form of XBP-1. The underlined regions of the sequence correspond to (or are reverse complements of) primers (SEQ ID NOs:8 and 9) for amplifying a region of the human XBP-1 mRNA that includes a splice junction. The splice junction is between nucleotides 5 506 and 507. The bold, underlined regions of the amino acid sequence is the sequence of the C-terminal portion of the protein encoded by the spliced form (SEQ ID NO: 6) that differs from that encoded by the unspliced form, which is bold and underlined in Figure 6.

FIG. 6 is text illustrating the mRNA and amino acid sequences for the unspliced form of XBP-1. The underlined regions of the sequence correspond to (or are reverse complements of) primers (SEQ ID NOs:8 and 9) for amplifying a region of the human XBP-1 mRNA that includes 10 a splice junction. The boxed region of the nucleotide sequence is the sequence spliced out by IRE1 (SEQ ID NO: 5). The splice junction is between nucleotides 506 and 507 in Figure 5. The bold, underlined regions of the amino acid sequence is the sequence of the C-terminal portion of the protein encoded by the unspliced form (SEQ ID NO: 7) that differs from that encoded by the 15 spliced form, which is bold and underlined in Figure 5.

FIG. 7 is a graph illustrating the standard curve for amplification of the spliced XBP-1 target detected using a cybergreen-labeled probe. Ct is the threshold cycle.

FIG. 8 is a graph illustrating the standard curve for amplification of the unspliced XBP-1 target detected using a cybergreen-labeled probe. Ct is the threshold cycle.

FIG. 9 is a Western blot analysis of wild-type and kinase inactive K599A (IRE1aKA) 20 human IRE1a expressed in COS7 cells using PIRE1A1 antibody (P- IRE1a) or total IRE1a antibody. PIRE1A1 antibody specifically detects wild-type IRE1a, which is known to be autophosphorylated by over-expression.

FIGs. 10A-10F are a series of pseudocolored photomicrographs showing 25 immunocytochemical staining of COS7 cells expressing Flag-tagged human wild-type (A-C) or P724L WFS1 (D-F). Staining with anti-Flag monoclonal antibody shows the distribution of wild-type or P724L WFS1 protein (A and D). Staining of the same cells with anti-ribophorin I antibody shows the structure of the ER (B and E). Merged images show the co-localization of WFS1 and ribophorin I (C and F). Bars: 10 mM.

FIG. 11A is a Western blot showing the effect of coexpression of ubiquitinK48R on the 30 expression level of wild-type or P724L WFS1. Lanes 1 and 3: COS7 cells transfected with

wild-type or P724L WFS1 expression vector alone. Lanes-2 and 4: COS7 cells cotransfected with HA-tagged ubiquitinK48R (UbK48R) expression vector.

FIG. 11B is a Western blot showing the results of immunoprecipitation of ubiquitin immunoreactive polypeptides with anti-WFS1 antibody. Fibroblasts from an unaffected individual (control) and a patient with Wolfram syndrome (WFS) were lysed in detergent. Cells were treated (+) or untreated (-) with MG132 (2 mM) for 16 hours. Detergent-soluble fractions were immunoprecipitated by anti-WFS1 antibody, separated on 4-20% linear gradient SDS-PAGE and immunoblotted with anti-ubiquitin antibody.

FIG. 11C is a Western blot showing high-molecular-weight complexes of WFS1P724L in detergent-insoluble fractions. COS7 cells transfected with Flag-tagged wild-type or P724L WFS1 expression vector were separated into detergent-soluble (upper panel) and detergent-insoluble (lower panel) fractions and immunoblotted with anti-Flag antibody.

FIG. 12A is a Western blot showing ubiquitination of WFS1 by EDEM. COS7 cells were cotransfected with Flag-tagged wild-type or P724L mutant WFS1, Myc-tagged EDEM, and HA-tagged ubiquitin. Cells were lysed in detergent, immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-HA antibody.

FIG. 12B is a Western blot showing the association of EDEM with mutant WFS1. COS7 cells were co-transfected with Flag-tagged wild-type or P724L WFS1 and Myc-tagged EDEM. Lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc antibody.

FIG. 12C is a bar graph illustrating that EDEM is upregulated in lymphocytes from WFS patients. Quantitative real-time PCR of reverse transcribed RNA of lymphoblast cells from Wolfram syndrome patients (WFS), their relatives who are heterozygous for the WFS1 mutation (Hetero), and the relatives who are homozygous normal. The amount of EDEM mRNA was normalized to the amount of GAPDH mRNA in each sample (n=8, values are mean ± s.e.m.)

FIGs. 13A-D are four bar graphs illustrating the results of quantitative real-time PCR of WFS1 using reverse-transcribed RNA from wild-type (WT) and *Ire1α* knock-out (*Ire1α*/-) mouse embryonic fibroblast cells. Cells were untreated or treated with tunicamycin (TM) (13A and B), thapsigargin (TG) (13C) or dithiothreitol (DTT) (13D) for six hours. EDEM expression by TM was also shown as control (13B). The amount of mouse WFS1 and EDEM mRNA was normalized to the amount of GAPDH mRNA in each sample.

DETAILED DESCRIPTION

Since the ER stress signaling network plays a role in the pathogenesis of human diseases, it is important to monitor the ER stress level in mammalian cells. The present invention provides methods and reagents to quantify ER stress levels.

5 IRE1 is one of the most upstream components of ER stress signaling network and it is a sensor for ER stress. The present invention features quantifying IRE1 activation levels as a measure of ER stress. Because it is difficult to measure IRE1 activation levels directly, XBP-1 mRNA splicing levels, which precisely reflect IRE1 activation, are used to quantify the IRE1 activation levels. Spliced XBP-1 mRNA encodes the active XBP-1 transcription factor and activates the UPR. The invention features methods to quantify the activation level of XBP-1 10 using Reverse Transcriptase-PCR (RT-PCR). Primers are designed to amplify the region encompassing the splice junction of XBP-1 mRNA. The spliced form (the active form) of XBP-1 mRNA (cDNA) is smaller than the unspliced form (inactive form) by 26 base pairs. The size difference between the two forms can be visualized, for example, by electrophoresing the 15 PCR products on an agarose gel.

Various aspects of the invention are described in further detail in the following subsections.

I. ER Stress and ER Stress Signaling Pathway Assays

20 The unfolded protein response (UPR) is a cellular adaptive response that counteracts ER stress. The UPR includes three different pathways to address ER stress: (1) gene expression, (2) translational attenuation, and (3) protein degradation. Inositol Requiring 1 (IRE1), an ER-resident transmembrane protein kinase, is one of the furthest upstream components of the UPR, and acts as a central regulator for UPR-specific downstream gene expression and apoptosis. At 25 least in part, IRE1 acts by splicing a small intron from XBP-1 mRNA.

IRE1 and XBP-1 are crucial components of the UPR, and the expression levels of the active forms of XBP-1 and IRE1 can serve as markers for ER stress levels. It is difficult to directly measure the activation level of IRE1, because although activation of IRE1 by phosphorylation causes a shift to lower mobility on an SDS-polyacrylamide gel, the shift is very 30 small and thus difficult to detect. To overcome this difficulty, the new methods use XBP-1 as a measure of ER stress level.

XBP-1 mRNA splicing levels can be detected using any method known in the art, e.g.,
Northern blotting, *in situ* hybridization (Parker & Barnes 1999 Methods in Molecular Biology
106:247–283), RNase protection assays (Hod 1992, Biotechniques 13:852–854; Saccomanno et
al. 1992 Biotechniques 13: 846–85), or reverse transcription polymerase chain reaction (RT-
PCR) (Weis et al. 1992 Trends in Genetics 8:263–264).

In some embodiments, splice levels are detected using a nucleic acid probe, e.g., a
labeled probe (a number of suitable labels are known in the art, including radioactive,
fluorescent, spin, and calorimetric labels), that hybridizes to the intron that is removed from the
XBP-1 sequence by splicing.

In some embodiments, XBP-1 splicing is detected using RT-PCR (reverse transcription-
polymerase chain reaction, typically involving cDNA synthesis from a target mRNA by reverse
transcription, followed by PCR amplification) and a pair of primers designed to amplify a region
including the splice site. RT PCR methods are known in the art.

In some embodiments, the methods described herein measure splicing of XBP-1 by RT-
PCR, optionally followed by Pst I digestion (See Examples 2-4). The mRNA and amino acid
sequences for the spliced and unspliced forms of XBP-1 are shown in Figures 5 and 6,
respectively. The underlined regions of each sequence correspond to (or are reverse
complements of) primers for amplifying a region of the human XBP-1 mRNA that includes a
splice junction. Additional primer pairs can readily be designed by the skilled artisan given the
above sequences and primer design programs. The boxed region of the nucleotide sequence in
Figure 6 is the sequences spliced out by IRE1. The splice junction is between nucleotides 506
and 507 in Figure 5. The bold, underlined regions of the amino acid sequence in Figure 5 is the
sequence of the protein encoded by the spliced form that differs from that encoded by the
unspliced form, which is bold and underlined in Figure 6.

In some embodiments, real-time PCR, e.g., as described in Bustin et al., J. Mol.
Endocrinol. (2000) 25, 169–193, is used, for example, when more accurate quantification of
splicing levels is required, e.g., where splicing levels are neither very high (e.g., most of the
XBP-1 is in spliced form) nor very low (e.g., only some of the XBP-1 is in spliced form), but are
in between (e.g., there is a more nearly balanced mixture of spliced and non-spliced XBP-1).

As noted above, any pairs of primers that can amplify the region of the target XBP-1
mRNA that includes a splice junction can be used. Exemplary sequences for primers are

provided herein. Typically, the primer set will include a first primer that is identical to or complementary to a sequence that is 5' of the spliced intron region, and a second primer that is identical to or complementary to a sequence that is 3' of the spliced intron region, such that when the two primers are used in a polymerase chain reaction, a region of suitable size is obtained.

- 5 One of skill in the art will be able to design a suitable set of primers using the sequences of XBP-1 known in the art and provided herein.

In some embodiments, levels of ER stress are detected using a binding agent specific for the spliced or unspliced form of XBP-1 protein. In some embodiments, the binding agent is an antibody that is specific for the spliced or unspliced form, e.g., recognizes an epitope that is 3' of the splice site. For example, an antibody that is specific for the spliced form can recognize an epitope in SEQ ID NO:6; an antibody specific for the unspliced form can recognize an epitope in SEQ ID NO: 7. Such antibodies can include any form-specific antibody (e.g., a monospecific, or a recombinant or modified antibody), and includes antigen-binding fragments thereof (e.g., Fab, F(ab')₂, Fv or single chain Fv fragments). The antibodies can be of the various isotypes, including: IgG (e.g., IgG₁, IgG₂, IgG₃, IgG₄), IgM, IgA₁, IgA₂, IgD, or IgE. The antibody molecules can be full-length (e.g., an IgG₁ or IgG₄ antibody) or can include only an antigen-binding fragment (e.g., a Fab, F(ab')₂, Fv or a single chain Fv fragment). These include monoclonal antibodies, recombinant antibodies, chimeric antibodies, humanized antibodies, deimmunized antibodies, as well as antigen-binding fragments of the foregoing.

- 20 Antibodies (e.g., monoclonal antibodies from differing organisms, e.g., rodent, sheep, human) can be produced using art-recognized methods. Once the antibodies are obtained, the variable regions can be sequenced. The location of the CDRs and framework residues can be determined (see, Kabat, E.A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) J. Mol. Biol. 196:901-917). The light and heavy chain variable regions can, optionally, be ligated to corresponding constant regions. A light and the heavy immunoglobulin chains can be generated and co-expressed into the appropriate host cells.

- 25 Monoclonal antibodies can be used in the methods described herein. Suitable monoclonal antibodies can be generated using techniques known in the art. Monoclonal antibodies can be produced by a variety of techniques, including conventional monoclonal antibody methodology e.g., the standard somatic cell hybridization technique of Kohler and

Milstein, Nature 256: 495 (1975). See generally, Harlow, E. and Lane, D. (1999) Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed e.g., viral or oncogenic transformation of B lymphocytes. A typical animal system for preparing hybridomas is the murine system.

5 Hybridoma production in the mouse is a well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

Human monoclonal antibodies (mAbs) directed against human proteins can be generated 10 using transgenic mice carrying the human immunoglobulin genes rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. International Application WO 92/03918; Kay et al. 15 International Application 92/03917; Lonberg, N. et al. 1994 Nature 368:856-859; Green, L.L. et al. 1994 Nature Genet. 7:13-21; Morrison, S.L. et al. 1994 Proc. Natl. Acad. Sci. USA 81:6851-6855; Bruggeman et al. 1993 Year Immunol 7:33-40; Tuallon et al. 1993 PNAS 90:3720-3724; Bruggeman et al. 1991 Eur J Immunol 21:1323-1326). Thus, the invention includes antibodies specific for a spliced or unspliced form of XBP-1, and for the autophosphorylated form of IRE1.

20 Useful immunogens for the purpose of producing anti-XBP-1 antibodies include peptides comprising portions of XBP-1 that are unique to either the spliced or unspliced form of XBP-1, e.g., all or part of the sequences shown in SEQ ID NOs:6 (spliced form) and 7 (unspliced form). Useful immunogens for the purpose of producing antibodies specific for the autophosphorylated form of IRE1 include phosphopeptides comprising the sequence surrounding the 25 autophosphorylation site, wherein the autophosphorylation site is phosphorylated (e.g., see Example 6).

The antibodies can be labeled to facilitate detection and quantification of XBP-1 splicing or IRE1 autophosphorylation levels. Numerous suitable labels, and methods for labeling the 30 antibodies, are known in the art. Examples of suitable labels include a fluorescent label, a biologically active enzyme label, a radioisotope (e.g., a radioactive ion), a nuclear magnetic

resonance active label, a luminescent label, or a chromophore. In some embodiments, a labeled secondary antibody is used. See, e.g., Harlow and Lane, *Id.*

Quantitation can be performed using any method known in the art, including but not limited to fluorometry, gamma counting, scintillation counting, spectrophotometry, kinetic phosphorescence, or phosphorimaging. Computer-based methods can be used to facilitate analysis.

Protein Conformation Diseases and ER Stress Disorders

Mutations in integral membrane proteins, such as the cystic fibrosis transmembrane conductance regulator protein, are known to cause the accumulation of misfolded proteins in the ER, which, in turn, causes a particular type of intracellular stress termed ER stress (Harding et al., (2002) *Annu Rev Cell Dev Biol* 18, 575-599). Accumulating evidence suggests that a high level of ER stress or defective ER stress signaling causes β -cell death in the development of diabetes (Harding and Ron (2002) *Diabetes* 51 Suppl 3, S455-461). The unfolded protein response (UPR) is an intracellular stress management system that counteracts ER stress (Harding et al., (2002) *Annu Rev Cell Dev Biol* 18, 575-599; Kaufman et al., (2002) *Nat Rev Mol Cell Biol* 3, 411-421; Mori, K. (2000) *Cell* 101, 451-454). The UPR has three components: gene expression, translational attenuation, and ER-associated protein degradation (the ERAD system). The ERAD system has an important function in the survival of ER stressed cells.

Wolfram Syndrome

Wolfram syndrome was first reported in 1938 by Wolfram and Wagener (Wolfram, D. J., and Wagener, H. P. (1938) *May Clin Proc* 1, 715-718), who analyzed four siblings with the combination of juvenile diabetes and optic atrophy. Because a significant portion of patients with Wolfram syndrome develop diabetes insipidus and auditory nerve deafness, this syndrome is also referred to as the diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (DIDMOAD) syndrome (Barrett and Bundey (1997) *J Med Genet* 34, 838-841; Rando et al., (1992) *Neurology* 42, 1220-1224). Its pathogenesis is still unknown. Patients with Wolfram syndrome do not have either insulitis or obesity. However, β -cells in pancreatic islets are selectively destroyed (Karasik et al., (1989) *Diabetes Care* 12, 135-138). The mechanism of β -cell death seen in Wolfram syndrome patients may be the same as, or similar to, the accelerated form of cell death seen in type-2 diabetes patients. Families that exhibit Wolfram syndrome share mutations in a gene encoding WFS1 protein, a trans-membrane protein in the

endoplasmic reticulum (ER) (Inoue et al., (1998) *Nature Genetics* 20, 143-148; Strom et al., (1998) *Hum Mol Genet* 7, 2021-2028). Most of the WFS1 mutations in Wolfram syndrome patients occur in exon 8, including the P724L mutation.

As described herein (see Examples 7-10), the mutant WFS1 protein seen in patients with Wolfram syndrome accumulates in the ER and activates its associated system for degrading mutant proteins in the endoplasmic reticulum. In lymphoblast cells from patients with Wolfram syndrome, expression of endoplasmic reticulum degradation-enhancing alpha-mannosidase-like protein, a central component of the protein degradation system, is significantly upregulated. In addition, we show that mutant WFS1 protein tends to form insoluble aggregates that are not degraded by this system. These results indicate that the pathogenesis of Wolfram syndrome involves the combined effects of a lack of functional WFS1 protein and the presence of insoluble WFS1 aggregates in cells. Thus, the methods described herein can be used to identify potential therapeutic agents for the treatment of Wolfram Syndrome.

15 II. Uses

Quantifying or detecting ER stress is useful in any situation where it is suspected or has been determined that such stress may regulate a normal cellular phenotype (e.g., regulate apoptosis) or cause or contribute to a disease phenotype (e.g., a protein conformational disease phenotype). In mammalian cells, ER stress is regulated, at least in part, by an ER stress signaling pathway. This pathway is an evolutionarily conserved signaling network that is emerging as the major quality controller of newly synthesized proteins.

ER stress signaling is likely to be crucial for protein secretion and the development of secretory cells, such as plasma cells, adipocytes, and trophoblast cells in placenta. These data also suggest that defects in this signaling network can cause or contribute to human diseases, such as the diseases listed in Table 1, as well as others, including some forms of juvenile diabetes, inflammatory bowel disease, and cancers originated from secretory cells (e.g., breast cancer and prostate cancer).

The methods and reagents of the invention are suitable for use in methods to further study the role of ER stress in cellular processes such as apoptosis and contribution of such processes in a variety of ER stress diseases, and in methods of screening for compounds, e.g., drugs, useful in the treatment of such diseases. Thus, in some embodiments, the methods include providing a ER

stress model system, e.g., a cell or animal model of an ER stress disease; optionally increasing levels of ER stress in the cells or animal (e.g., in at least some of the cells of the animal); contacting the cells with a test compound; and evaluating the levels of XBP-1 splicing in the cells in the presence and absence of the test compound, thus evaluating the effect of the 5 compound on ER stress. Those compounds that produce a desired effect on ER stress, e.g., that substantially reduce ER stress (i.e., as measured by XBP-1 splicing levels), e.g., by at least about 20%, e.g., about 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, can be considered as candidate compounds and further evaluated for therapeutic activity using methods known in the art, e.g., administering the candidate compounds to an animal, e.g., an animal model of an ER 10 stress disease, and evaluating an effect of the compound on the animal, e.g., therapeutic efficacy or toxicity.

In some embodiments, the system is an animal model of an ER stress disease, e.g., an animal model of diabetes (e.g., type 1 or type 2 diabetes), Alzheimer's disease, Parkinson's disease, Cystic Fibrosis, Wolfram syndrome, familial hypercholesterolaemia and alpha1 15 antitrypsin deficiency, or cells derived therefrom. Typically an ER stress disease can be induced in an otherwise healthy animal or cells by administering a compound known to cause ER dysfunction, e.g., by administering a sublethal dose of thapsigargin, tunicamycin (e.g., 0.25 – 1 mg/kg tunicamycin; see Zinszner et al., Genes and Dev. 1998, 12:982-995), or a proteosome inhibitor, e.g., lactacystin.

The new methods can be used in high-throughput screening methods, e.g., to screen a 20 library of test compounds, e.g., to identify candidate therapeutic agents for use in the treatment of ER stress disease, e.g., to identify candidate agents for the treatment of diabetes (e.g., type 1 or type 2 diabetes), Alzheimer's disease, Parkinson's disease, Cystic Fibrosis, Wolfram syndrome, familial hypercholesterolaemia, or alpha1 antitrypsin deficiency. For example, 25 antibody-based, fluorescence-based, or PCR-based high-throughput screening methods are known in the art and can be used to detect an effect on ER stress levels, e.g., by measuring IRE1 activation levels, for example, by measuring XBA-I splicing levels or IRE1 autophosphorylation levels.

As one example, illustrated in Example 5, an XBA-I/GFP fusion protein can be used to 30 detect splicing levels; GFP (or any other detectable, e.g., fluorescent or chromatogenic, peptide or polypeptide) is cloned at the C-terminal end of XBA-I lacking a stop codon, in-frame with a

spliced from of XBA-I. Since the splicing shifts the frame of the C-terminal portion of the protein, an active form of GFP will be produced only when spliced XBA-I is produced. This is a particularly useful measure of splicing as the ratio of GFP molecules will be 1:1 with spliced XBA-I molecules, and detecting the GFP signal directly measures the amount of spliced XBA-I.

5 As another example, the antibody described herein that binds specifically to the autophosphorylated form of IRE1 can be used to determine levels of ER stress by detecting levels of IRE1 autophosphorylation. A number of methods are known in the art for using antibodies in this fashion.

10 High throughput methods for detecting fluorescence in cells are known in the art, and a number of commercially available systems can be adapted for use, e.g., systems using microplate readers, including those developed and used by Aventis, Genetix, Acumen, and Millipore. For example, for high throughput screens, multi-well plates, e.g., plates with 96, 384, or more separate areas, e.g., wells, e.g., separated by a barrier, can be screened. Suitable plates are known in the art, and can be manufactured, modified, or are commercially available. In some 15 embodiments, each area, e.g., each well, contains a unique compound, e.g., small molecule of known or unknown structure, or a pool of molecules of known or unknown structure.

The test compound library can be a library of compounds of related or unrelated structures. Such libraries are known in the art and are commercially available or can be synthesized using methods known in the art.

20 Libraries of test compounds, such as small molecules, are available, e.g., commercially available, or can be synthesized using methods known in the art. As used herein, "small molecules" refers to small organic or inorganic molecules. In some embodiments, small molecules useful for the invention have a molecular weight of less than 10,000 Daltons (Da). The compounds can include organic or inorganic naturally occurring or synthetic molecules 25 including but not limited to soluble biomolecules such as oligonucleotides, polypeptides, polysaccharides, antibodies, fatty acids, etc.

The compounds can be natural products or members of a combinatorial chemistry library. A set of diverse molecules should be used to cover a variety of functions such as charge, aromaticity, hydrogen bonding, flexibility, size, length of side chain, hydrophobicity, and rigidity. 30 Combinatorial techniques suitable for synthesizing small molecule compounds are known in the art, e.g., as exemplified by Obrecht, D. and Villalgrodo, J.M., Solid-Supported Combinatorial

and Parallel Synthesis of Small-Molecular-Weight Compound Libraries, Pergamon-Elsevier Science Limited (1998), and include those such as the “split and pool” or “parallel” synthesis techniques, solid-phase and solution-phase techniques, and encoding techniques (see, for example, Czarnik, A.W., *Curr. Opin. Chem. Bio.*, (1997) 1, 60). In addition, a number of compound, e.g., small molecule, libraries are commercially available.

Libraries and test compounds screened using the methods of the present invention can comprise a variety of types of compounds. A given library, for example, can comprise a set of structurally related or unrelated test compounds. In some embodiments, the compounds and libraries thereof can be obtained by systematically altering the structure of a first compound, e.g., a small molecule, e.g., using methods known in the art or the methods described herein, and correlating that structure to a resulting biological activity, e.g., a structure-activity relationship study. As one of skill in the art will appreciate, there are a variety of standard methods for creating such a structure-activity relationship. Thus, in some instances, the work may be largely empirical, and in others, the three-dimensional structure of an endogenous polypeptide or portion thereof can be used as a starting point for the rational design of a test compound or compounds, e.g., a small molecule. For example, in one embodiment, a general library of small molecules is screened using the methods described herein.

Compounds identified as “hits” (e.g., compounds that decrease ER stress) in the first screen can be selected and systematically altered, e.g., using rational design, to optimize binding affinity, avidity, specificity, or other parameter. Such optimization can also be screened for using the methods described herein. Thus, in one embodiment, the invention includes screening a first library of compounds using the methods described herein, identifying one or more hits in that library, subjecting those hits to systematic structural alteration to create additional libraries of compounds structurally related to the hit, and screening the second library using the methods described herein.

In some embodiments, each well contains one or more unique test compounds, e.g., small molecules that are different from the test compounds in at least one of the other wells. In some embodiments, the multi-well plate also includes one or more positive and/or negative control wells. Negative control wells can contain, for example, no test compound other negative control. Positive control wells can contain, for example, compounds known to inhibit ER stress. In some embodiments, a number of multi-well plates, each comprising a unique set of small molecules,

are screened. In this way, a library of test compounds in the hundreds, thousands, or millions can be screened for identification of ER stress reducing molecules.

The methods of the invention are also suitable for use in methods of diagnosing ER stress diseases, e.g., as described herein. For example, the methods and reagents can be used for 5 diagnosing diabetes, e.g., Type 2 diabetes or certain forms of Type 1 diabetes, e.g., Wolcott-Rallison syndrome and Wolfram syndrome, as these diseases are believed to be caused, at least in part, by increased ER stress. More than one million people suffer from type 1 diabetes in the U.S. In this disease, insulin production is abnormally low because beta-cells in pancreatic islets are destroyed. Recent observations in the Akita diabetes model mouse (a C57BL/6 mouse with a 10 mutation in insulin 2 gene; Oyadomari et al., J. Clin. Inv. 109:525-32 (2002); Urano et al., Science 287:664-6 (2000)) support the hypothesis that sufficient endoplasmic reticulum (ER) stress can cause beta-cell death.

It is believed that defects in the ER stress signaling network also cause or contribute to 15 human diseases including many of the diseases listed in Table 1, as well as others, including inflammatory bowel disease, and cancers originated from secretory cells (e.g., breast cancer and prostate cancer), as well as Parkinson's disease, cystic fibrosis, familial hypercholesterolaemia, alpha1-antitrypsin deficiency, and Alzheimer's disease. Thus, it is contemplated that the ER stress measurement methodologies described herein will also be useful in methods for diagnosing any of these diseases in patients. In some embodiments, the methods and reagents 20 described herein can be used to diagnose the stage of a disease in patients. In some embodiments, the disease is multiple myeloma. Multiple myeloma is a cancer of plasma cells. ER stress signaling is important for the development of plasma cells. Thus, it is expected that ER stress levels will be very high in multiple myeloma cells, and higher stress levels is likely to correlate to more aggressive disease.

25

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

Example 1: XBP-1 Splicing Assay

RNA from cells was reverse transcribed using Oligo-dT primer. PCR is performed using primers shown in Table 2.

5

Table 2. RT-PCR primers

Species	Sense (S) or Antisense (AS)	Sequence	SEQ ID NO:
Human	hXBP-1.1S	AAACAGAGTAGCAG <u>CT</u> CAGACTGC	8
Human	hXBP-1.2AS	TGGCAGTAGGCTGGATGAAAGC	9
Mouse	mXBP-1.3S	AAACAGAGTAGCAG <u>CG</u> CAGACTGC	10
Mouse	mXBP-1.6AS	CAGACAATGGCTGGATGAAAGC	11
Rat	rXBP-1.3S	AAACAGAGTAGCAG <u>CA</u> CAGACTGC	12
Rat	mXBP-1.6AS	CAGACAATGGCTGGATGAAAGC	13

These primers amplify a 768-base pair PCR product for human, a 774-base pair PCR product for mouse, and a 774-base pair PCR product for rat from the unspliced XBP-1, and 742-base pair (human) and 748-base pair (mouse, rat) PCR products from the spliced form. These primers were designed to amplify the region encompassing the splice junction of XBP-1 mRNA.

Reverse Transcriptase-PCR (RT-PCR) was performed using mRNA isolated using standard methods from a wild-type mouse fibroblast cell line and *Ire1α*:*Ire1β* double knock-out cell line. The cells were treated with tunicamycin or thapsigargin for 4 or 8 hours. Tunicamycin causes ER stress experimentally by blocking N-linked glycosylation, which is a crucial step for protein folding in the ER. Thapsigargin also induces ER stress experimentally by altering Calcium ion concentrations in the ER.

The results are illustrated in Figure 1B. The 26 base pair size difference between the two forms, spliced and unspliced, was visualized by running the PCR product on 2.5% agarose gel (Figure 1B). The thermal cycle reaction was performed as follows: 94°C for 3 minutes, followed by 35 cycles at 94°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute, and 72°C for 10 minutes. RT-PCR analysis detected predominantly smaller fragment corresponding to active form (spliced form) of XBP-1 mRNA in wild-type cell line treated with tunicamycin or thapsigargin. In contrast, the same analysis detected only inactive form of XBP-1 mRNA in *Ire1α*-/-:*Ire1β*-/- double knock-out cell line (Figure 1B).

Example 2: XBP-1 Splicing Assay with Pst I Digestion

A Pst I restriction site is removed by IRE1-mediated cleavage and splicing of the mRNA, thus, the results of the experiment described in Example 1 can also be achieved using an intermediate step of Pst I cleavage to facilitate distinguishing between spliced and unspliced XBP-1. Pst I digestion of the spliced form of XBP-1 yields a 768-base pair fragment for human, 774-base pair fragment for mouse and rat. The unspliced forms of XBP-1 yield 285 base pair and 483 base pair fragments for human, 291 base pair and 483 base pair fragments for mouse and rat.

RT-PCR performed as described in Example 1 was followed by Pst I digestion, and the digested products were visualized on a 2% agarose gel. Since the intron removed by IRE1-mediated splicing contains the Pst I site, the spliced form (the active form) of XBP-1 mRNA (cDNA) loses its Pst I site after IRE1 processing. Pst I digestion of RT-PCR product produces undigested larger fragment corresponding to the active form (spliced form, no Pst I site) of XBP-1 mRNA and two smaller, digested fragments corresponding to the inactive form (unspliced form, which retains the Pst I site) (Figure 2A). Pst I digestion of RT-PCR product generated as described above detected predominant non-digested fragment corresponding to active form (spliced form) of XBP-1 mRNA in wild-type cell line treated with tunicamycin or thapsigargin. In contrast, the same analysis detected only inactive form of XBP-1 mRNA in *Irel1 α -/-:Irel1 β -/- double knock-out cell line (Figure 2B).*

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Example 3: ER stress Signaling is Activated in Islet Cells under Physiological Conditions

To determine whether ER stress signaling is activated in islet cells under physiological conditions, XBP-1 splicing was monitored in freshly isolated mouse islet cells, using the methods described above in Example 2. The results are shown in Figure 3. High levels of XBP-1 mRNA splicing were detected in the islet cells. Dithiothreitol (DTT) treatment enhanced the XBP-1 splicing. It is known that DTT blocks disulfide bond formation experimentally, resulting in ER stress. These results illustrate that XBP-1 splicing, and hence ER stress, occurs in islet cells under physiological conditions. This demonstrates that the methods described herein can be successfully used to detect and measure ER stress under physiological conditions; in addition, as the islet cells secrete insulin, this demonstrates that ER stress may play a role in the etiology of diabetes.

Example 4: Insulin-2 Mutation in the Akita Mouse Causes ER Stress in MIN6 Cells

The Akita diabetes model mouse is a C57BL/6 mouse which is heterozygous for a mutation in insulin 2 gene. This mutation results in an amino acid substitution, cysteine 96 to tyrosine. Cysteine 96 is involved in the formation of one of the two disulfide bonds between the A and B chains of mature insulin. It is likely that this mutation causes incorrect folding of insulin precursor in the endoplasmic reticulum (ER) of pancreatic beta-cells. Diabetes in the Akita mouse is accompanied by neither obesity nor insulitis. These mice spontaneously develop diabetes with dramatic reduction in beta-cell mass. Symptoms include hyperglycemia, hypoinsulinemia, polydipsia, and polyuria, beginning around 4 weeks of age. This condition in the Akita mouse is termed diabetes.

Since the phenotype of the Akita mouse is caused by a mutation which can cause conformational changes in the insulin 2 (Ins2) gene product (Wang et al., J., 1999. J. Clin. Invest. 103:27-37), it is hypothesized that pancreatic cells in Akita mice are under ER stress, and this stress can cause beta cell death. To test this hypothesis, XBP-1 splicing levels were measured in mouse insulinoma cells (MIN6 cells) expressing either an Ins2 gene with the Akita mutation or a wild-type insulin-2 gene. The MIN6 cells were cultured in 10 cm collagen-coated dishes in DMEM supplemented with 25 mM glucose and 15% FCS. Plasmids encoding the wild-type or mutant Ins-2 genes were transfected into the cells using Fugene™ transfection reagent following the manufacturer's instructions (Roche, Basel, Switzerland).

The results are shown in Figure 4. High XBP-1 splicing levels, which reflected high ER stress levels, were detected in the MIN6 cells expressing mutant insulin 2 gene. This indicates that the methods described herein can be used to detect differences in ER stress levels correlating with disease states.

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Example 4: XBP-1 Splicing Assay Using Quantitative Real-Time PCR

This example describes a method to quantify the expression levels of spliced form and unspliced form of XBP-1 mRNA using real-time PCR. Briefly, RNA from cells was reverse transcribed using Oligo-dT primer. PCR was performed using primers shown in Table 3.

Table 3: Real-Time PCR primers

Species of Target	Sequence	Seq. Name	SEQ ID NO:
Human	CAG CAC TCA GAC TAC GTG CA	hXBP1.3S:	
	ATC CAT GGG GAG ATG TTC TGG	hXBP1.6AS:	
	CTG AGT CCG AAT CAG GTG CAG	mXBP1.11S:	
Mouse	CAG CAC TCA GAC TAT GTG CA	mXBP1.7S	
	GTC CAT GGG AAG ATG TTC TGG	mXBP1.10AS	
	CTG AGT CCG AAT CAG GTG CAG	mXBP1.11S	
Rat	ATC CAT GGG AAG ATG TTC TGG	rXBP1.6AS	
	CAG CAC TCA GAC TAC GTG CG	rXBP1.7S	
	CTG AGT CCG AAT CAG GTG CAG	mXBP1.11S	

To amplify the active form of XBP-1 mRNA, mXBP1.11S and hXBP1.6AS (human target), mXBP1.11S and mXBP1.10AS (mouse target) and mXBP1.11S and rXBP1.6AS (rat target) were used. Two mismatches to the native XBP-1 sequence were introduced in the mXBP1.11S primer to reduce background signal.

To amplify the inactive form of XBP-1, hXBP-1.3S and hXBP1 (human target), mXBP1.7S and mXBP1.10AS (mouse target), and rXBP1.7S and rXBP1.6AS (rat target) were used.

The results using mouse XBP-1 cDNA as a target are illustrated in Figures 7 and 8. The thermal cycle reaction was performed using ABI prism 7000 sequencer detection system as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Standard curves for the amplification of the XBP-1 target detected using a cybergreen-labeled probe are shown in Figs. 7 and 8. Ct is the threshold cycle. The threshold cycle is when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase.

Example 5: XBP-1 Splicing Assay Using XBP-1-GFP Fusion Protein

XBP-1 splicing has also been detected using an XBP-1-GFP fusion protein. Briefly, human XBP-1 partial cDNA (without the stop codon) was cloned into pEGFP-N1 (CLONTECH). Under ER stress conditions, the EGFP is expressed as a fusion to the C-terminus of spliced XBP-1, because the spliced form is in the same reading frame as EGFP and there are no intervening stop codons. Under normal conditions, i.e., non-ER stress conditions, the EGFP is not expressed, as the EGFP is not in frame with the unspliced form of XBP-1.

Example 6: Anti-Phospho IRE1 α Antibodies

To directly quantify IRE1 activation levels, antibodies against the phosphorylated and non-phosphorylated forms of IRE1 α were generated. Peptide sequences used as immunogens to generate the antibodies are listed in Table 4. The phosphorylation site of Ire1a is conserved from lower eukaryotes to humans (Shamu and Walter, Embo J 15:3028-39 (1996); Tirasophon et al., Genes Dev 12:1812-24 (1998)).

Table 3. Peptide Sequences for Generating anti-Phospho IRE1 α antibody

Peptide Sequence	SEQ ID NO:	Antigen
CVGRH[pS]FSRRSG		Phospho IRE1 α
CVGRHSFSRRSG		IRE1"

The antibodies were produced using standard methodology. Briefly, the indicated phosphopeptides were synthesized, multi-link conjugated to KLH, and individually immunized following a 90-day protocol, using two specific pathogen free (SPF) rabbits. Four immunizations were performed per rabbit, with varying dosage. The antibody was prepared from bulk antiserum by affinity purification followed by adsorption against the non-phospho analog column peptide.

The specificity of the antibody, PIRE1A1 was tested by immunoblot analysis of wild-type or kinase inactive K599A human IRE1a expressed in COS7 cells (Figure 9). PIRE1A1 antibody specifically detects wild-type IRE1a which is known to be autophosphorylated by over-expression (3). PIRE1A1 antibody specifically detects the phosphorylated form of IRE1a protein. Immunoblot analysis of wild-type and kinase inactive K599A (IRE1aKA) human IRE1a expressed in COS7 cells using PIRE1A1 antibody (P- IRE1a) or total IRE1a antibody. As shown in Fig. 9, PIRE1A1 antibody specifically detects wild-type IRE1a which is known to be autophosphorylated by over-expression. The amount of total IRE1a is shown in the lower panel.

Example 7: Effect of the P724L Mutation in the Wolfram Gene WFS1 on Cellular Localization

The experiments in this Example evaluated the effect of the P724L mutation of WFS1 on cellular localization of wild-type and mutant WFS1.

Plasmids, cell culture, and transfection

Full-length human WFS1 cDNA and P724L mutant WFS1 cDNA was tagged with a Flag epitope and subcloned each to a pcDNA3 plasmid under the control of the cytomegalovirus

promoter using standard molecular biology methods. The P724L mutation was introduced using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA). COS7 cells were transfected using FuGene (Roche, Basel) and maintained in DMEM with 10% fetal bovine serum.

5 *Immunostaining*

Cells were fixed in 2% paraformaldehyde for 30 min at room temperature, then permeabilized with 0.1% Triton X-100 for 2 minutes. The fixed cells were washed with PBS, blocked with 10% BSA for 30 min, and incubated in primary antibody overnight at 4°C. The cells were washed 3 times in PBS/Tween™ 0.1% and incubated with secondary antibody for 1 hour at room temperature. Images were obtained with a Leica TCS SP2 AOBS Confocal Microscope with LCS Software.

10 Results:

The cellular localization of wild-type and mutant WFS1 was determined by immunostaining cells transfected with an expression vector for wild-type or P724L WFS1 tagged at its C-terminus with a Flag epitope. Immunostaining of cells expressing wild-type WFS1 with anti-Flag antibody showed a diffuse reticular pattern that co-localized with the ER marker ribophorin I (Fig. 10A-C). However, immunostaining of cells expressing mutant WFS1 with anti-Flag antibody showed a punctate staining pattern in the ER, suggesting that WFS1 tends to aggregate there (Fig. 10D-F). Part of WFS1^{P724L} showed a diffuse reticular pattern and was co-localized with ribophorin I, suggesting that this part of WFS1^{P724L} is localized to the ER membrane. However, the signal intensity of mutant WFS1 was much lower than that of wild-type WFS1 (Fig. 10D). These staining patterns suggest that in contrast to wild-type WFS1, most of the newly synthesized WFS1^{P724L} protein aggregates and thus is not expressed on the ER membrane.

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Example 8: Effect of the P724L Mutation in the Wolfram Gene WFS1 on Expression Levels, Ubiquitination and Aggregation

30 The experiments described in this Example evaluated the effect of the P724L mutation of WFS1 on expression levels, ubiquitination and aggregation of mutant WFS1.

Immunoblotting

The cells described in Example 7 were lysed in ice-cold buffer (20 mM Hepes, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1mM EDTA) containing protease inhibitors for 15 minutes on ice. Insoluble material was recovered by centrifugation at 13,000 g for 15 minutes and solubilized in 10 mM Tris-HCl and 1% SDS for 10 min at room temperature. After the 5 addition of 4 volumes of lysis buffer, samples were sonicated for 10 seconds. Lysates normalized for total protein (20 mg per lane) were separated using 4%-20% linear gradient SDS-PAGE (Bio Rad, Hercules, CA) and electroblotted.

Results:

Measuring the steady-state expression level of WFS1P724L by immunoblot analysis, we found that it did not accumulate to high levels in transfected cells, suggesting that WFS1P724L 10 was subject to increased intracellular degradation.

The WFS1P724L mutant was then co-expressed with a dominant negative form of ubiquitin to determine whether or not polyubiquitination is required for WFS1P724L degradation. The Lys-48 residue of ubiquitin, which is the site of isopeptide linkage of other 15 ubiquitin molecules, is essential for the formation of multi-ubiquitin chains. Mutant ubiquitin in which this invariant lysine is replaced by the arginine (K48R) is a polyubiquitin chain terminator that reduces the efficiency of proteasome-mediated degradation and stabilizes polyubiquitinated substrates (Chau et al., Science 243, 1576-1583 (1989); Finley et al., Mol Cell Biol 14, 5501-5509 (1994)). Co-expression of WFS1P724L and ubiquitinK48R increased the WFS1P724L 20 expression level as well as the wild-type WFS1 expression level (Fig. 11A), suggesting both are degraded by the ubiquitin-proteasome system.

To analyze the ubiquitination level of mutant WFS1 protein in Wolfram syndrome, detergent-soluble lysates were immunoprecipitated from the fibroblasts of a patient with this syndrome, using a polyclonal antibody to WFS1, then immunoblotted with a monoclonal 25 antibody to ubiquitin. The patient was a compound heterozygote for G695V and W648X. The W648X mutation predicts premature termination and a lack of 242aa of the C-terminus of WFS1 protein. Ubiquitin reactivity was increased in proteasome inhibitor MG132-treated cells and was higher in the patient's cells than in control cells (Fig. 11B), indicating that mutant WFS1 protein is more susceptible to ubiquitination than wild-type WFS1 protein.

30 The aggregation of WFS1P724L was assessed by SDS-PAGE immunoblot analysis of detergent-soluble and detergent-insoluble lysates from COS7 cells transiently expressing these

proteins. The formation of insoluble and high-molecular-weight complexes was much more prominent in cells expressing WFS1P724L than in cells expressing wild-type WFS1 (Fig. 11C, lower panel). This suggests that mutant WFS1 tends to misfold and form insoluble aggregates in the ER.

5 These results suggest that mutant WFS1 proteins in patients with Wolfram syndrome are rapidly degraded by the ubiquitin-proteasome pathway and that some of them form insoluble aggregates.

Example 9: Effect of the P724L Mutation in the Wolfram Gene WFS1 on Degradation

10

The experiments described in this Example evaluated the effect of the P724L mutation of WFS1 on degradation of mutant WFS1. EDEM is a type II ER transmembrane protein having homology to class I a1,2-mannosidase, which is involved in N-glycan processing (Hosokawa et al., EMBO Rep 2, 415-422 (2001)). It has been shown that EDEM is directly involved in the 15 ERAD system for glycoproteins (Hosokawa et al., 2001, supra; Hosokawa et al. J Biol Chem 278(28):26287-94 (2003); Molinari et al., Science 299, 1397-1400 (2003); Oda et al., Science 299, 1394-1397 (2003)). Because WFS1 is a glycoprotein localized to the ER, the involvement of EDEM in the degradation of WFS1P724L was evaluated.

15

Results:

20

To determine whether WFS1 is ubiquitinated by EDEM, Myc-tagged EDEM and either wild-type or P724L WFS1 was co-transfected with HA-tagged ubiquitin in COS7 cells. EDEM expression increased the ubiquitination of both wild-type and P724L WFS1. However, a higher level of ubiquitination occurred in cells expressing WFS1P724L than in cells expressing wild-type WFS1 (Fig. 12A). To test the association between WFS1 and EDEM, Myc-tagged EDEM and Flag-tagged WFS1P724L were co- transfected into COS7 cells, and these cells were subjected to co-immunoprecipitation analysis. Both wild-type and P724L WFS1 were associated with EDEM (Fig. 12B), suggesting that EDEM is involved in the degradation of WFS1 proteins. These results indicate that both wild-type and mutant WFS1 are degraded by the ERAD system, but that the mutant WFS1 is more susceptible to degradation by the EDEM-ERAD pathway.

25

30

To measure the activation level of the ERAD system in patients with Wolfram syndrome, quantitative real-time PCR was used to compare EDEM expression in lymphoblasts from patients and their relatives who were homozygous or heterozygous normal for the WFS1

mutation. As compared to patients' relatives who were homozygous normal, patients who were homozygous for the WFS1 mutation had 6 to 7 times higher average levels of EDEM messenger RNA, while patients' relatives who were heterozygous for this mutation had levels that were 4 to 5 times higher (Fig. 12C).

5 These findings indicate that the ERAD system is highly activated in patients with Wolfram syndrome.

Example 10: Effect of the P724L Mutation in the Wolfram Gene WFS1 on ER Stress

10 As noted above, WFS1 encodes an ER-resident transmembrane protein. Membrane proteins in the ER are often involved in the unfolded protein response (UPR), a system that mitigates intracellular stress caused by the accumulation of misfolded proteins in the ER (13,25). By measuring the expression level of WFS1 under ER stress, it has been found that WFS1 mRNA is induced by this stress and is under control of inositol requiring 1 (IRE1), a central component of the UPR (Fig. 13A-D). This suggests that WFS1 is also a component of the UPR and may be protective against ER stress.

Real-Time Polymerase Chain Reaction

20 Total RNA was isolated from the cells described in Example 7 by the guanidine-thiocyanate-acid-phenol extraction method, reverse transcribing 1 mg of total RNA from cells with Oligo-dT primer. For the thermal cycle reaction, the ABI prism 7000 sequencer detection system (Applied Biosystems, Foster City, CA) was used at 50°C for 2 min, 95°C for 10 min, then 40 cycles at 95°C for 15 sec and at 60° C for 1 min. The polymerase chain reaction (PCR) in triplicate for each sample and all experiments were repeated twice, using human GAPDH as a control. The following set of primers and Cyber Green (Applied Biosystems) for real-time PCR: for human endoplasmic reticulum degradation-enhancing alpha-mannidose-like protein (EDEM),
25 CAAGTGTGGGTACGCCACG (SEQ ID NO:) and AAAGAAGCTCTCCATCCGGTC (SEQ ID NO:); for mouse EDEM, CTACCTGCGAAGAGGCCG (SEQ ID NO:) and GTTCATGAG CTGCCCACTGA (SEQ ID NO:); and for mouse WFS1, CCATCAACATGCTCCCGTTC (SEQ ID NO:) and GGGTAGGCCTGCCATACA (SEQ ID NO:).

Results:

30 Quantitative real-time PCR of WFS1 using reverse-transcribed RNA from wild-type (WT) and Ire1a knock-out (Ire1a/-) mouse embryonic fibroblast cells. Cells were untreated or

treated with tunicamycin (TM) (Fig. 13A-B), thapsigargin (TG) (Fig. 13C) or dithiothreitol (DTT) (Fig. 13D) for six hours. EDEM expression in TM-treated cells was also shown as control (Fig. 13B). The amount of mouse WFS1 and EDEM mRNA was normalized to the amount of GAPDH mRNA in each sample.

5 The results described herein indicate that mutant WFS1 protein in patients with Wolfram syndrome forms insoluble high-molecular complexes that may be toxic to the cells. These findings suggest that the pathogenesis of Wolfram syndrome can be attributed to the combined effects of the lack of functional WFS1 protein and the presence of aggregated WFS1 proteins in cells.

10

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, 15 advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

- 1 1. A method of evaluating endoplasmic reticulum stress (ER stress) levels in a
2 cell or biological sample, the method comprising detecting an IRE1 activation level in
3 the cell or biological sample, wherein the IRE1 activation level correlates with ER
4 stress, thus evaluating the ER stress levels in the cell or biological sample.
- 5 2. The method of claim 1, wherein an IRE1 activation level is detected by
6 detecting an XBP-1 splicing level.
- 7 3. The method of claim 2, wherein the XBP-1 splicing level is determined by:
8 amplifying an XBP-1 mRNA region which includes a splice site, or portion
9 thereof; and
10 detecting the size of the amplified mRNA, wherein the size is indicative of
11 spliced or unspliced mRNA; such that the XBP-1 splicing level is determined.
- 12 4. The method of claim 3, wherein the amplified mRNA is subjected to
13 restriction enzyme digestion to facilitate detection of spliced or unspliced mRNA.
- 14 5. The method of claim 4, wherein the restriction enzyme digestion is Pst I
15 digestion.
- 16 6. The method of claim 1, wherein an IRE1 activation level is detected by
17 detecting the level of autophosphorylated IRE1.
- 18 7. The method of claim 1, wherein an IRE1 activation level is detected by
19 detecting the ratio of autophosphorylated to unphosphorylated IRE1.
- 20 8. The method of claim 6, wherein the level of phosphorylated IRE1 is detected
21 using an antibody that binds specifically to the autophosphorylated form of IRE1.
- 22 9. The method of any one of the preceding claims, wherein the ER stress level is
23 quantified in a cell.
- 24 10. The method of any one of the preceding claims, wherein the ER stress level is
25 quantified in a mammalian cell.
- 26 11. The method of any one of the preceding claims, wherein the ER stress level is
27 quantified in a human cell.

- 28 12. The method of claim 8, wherein the cell is a pancreatic beta cell.
- 29 13. The method of any one of claims 1-5, wherein the ER stress level is quantified
30 in a cell extract.
- 31 14. A method of diagnosing an ER stress disease in a subject, the method
32 comprising quantifying the level of ER stress in a cell or biological sample isolated
33 from the subject using a method according to any one of the preceding claims,
34 wherein an increased level of ER stress is indicative of an ER stress disease.
- 35 15. A method of monitoring the progression of an ER stress disease in a subject,
36 the method comprising quantifying the level of ER stress in a cell or biological
37 sample isolated from the subject at sequential time points using a method according
38 to any one of the preceding claims, wherein a change in level of ER stress is
39 indicative of the progress of the ER stress disease.
- 40 16. The method of claim 14 or 15, wherein the ER stress disease is diabetes.
- 41 17. A method of screening a test compound for an effect on ER stress, the method
42 comprising:
43 providing an ER stress model system (*e.g., a system comprising a cell*
44 *expressing IRE1 and XBP-1, e.g., a cultured cell or animal*);
45 optionally, increasing levels of ER stress in the system;
46 contacting the system with a test compound; and
47 evaluating the levels of IRE1 activation in the system in the presence
48 and absence of the test compound,
49 wherein an increase in IRE1 activation indicates an increase in ER stress, and a
50 decrease in IRE1 activation indicates a decrease in ER stress, thereby evaluating the effect of
51 the compound on ER stress.
- 52 18. The method of claim 17, wherein the ER stress model system is a cell or
53 animal model of an ER stress disease.
- 54 19. The method of claim 17, wherein increasing levels of ER stress in the system
55 comprises increasing levels of ER stress in the cell or in at least one cell in the
56 animal.

ABSTRACT

The present invention provides methods and reagents to quantify endoplasmic reticulum stress (ER stress) levels. Methods for quantifying ER stress in mammalian cells are exemplified.

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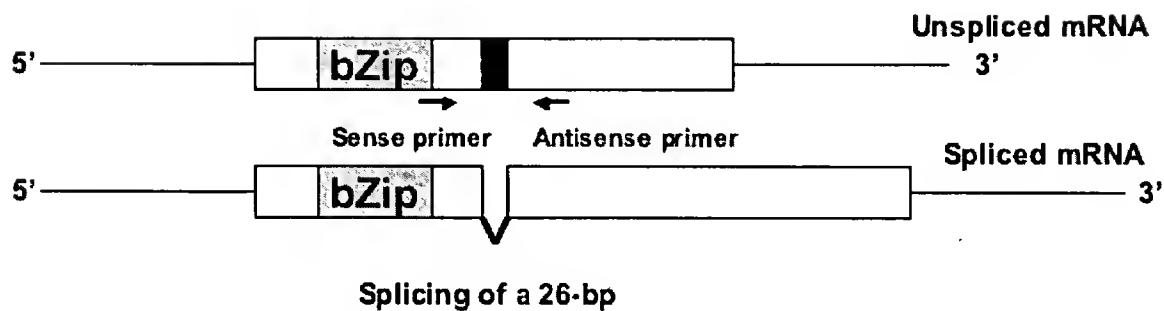


Figure 1B.

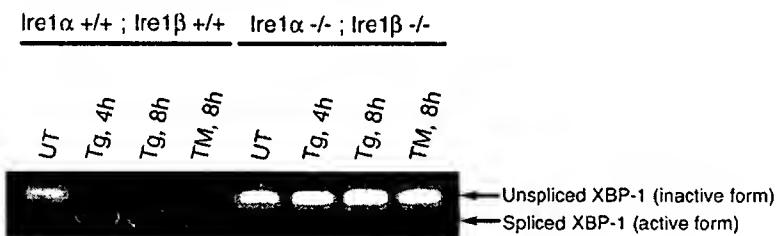


Figure 2A.

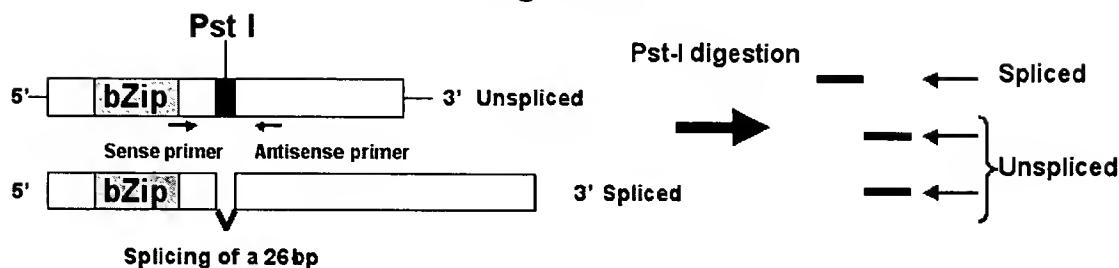


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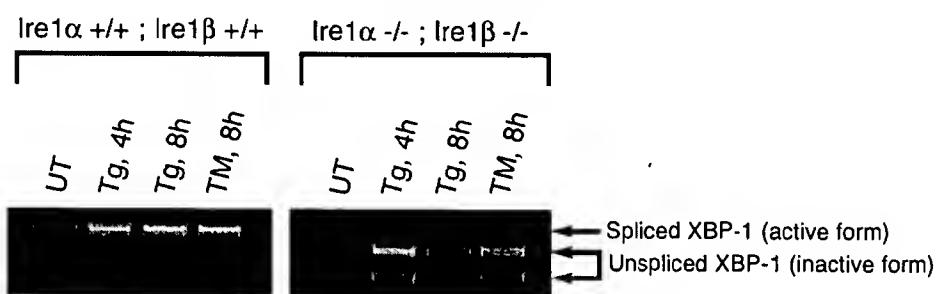


Figure 3.

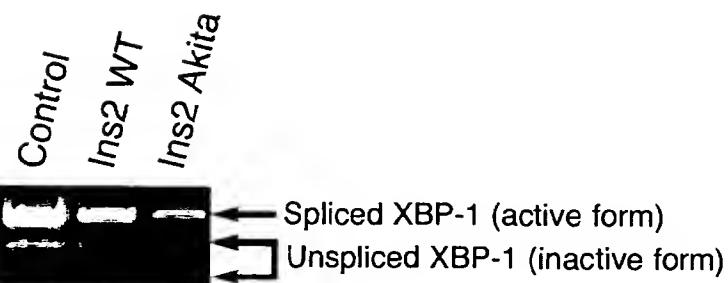
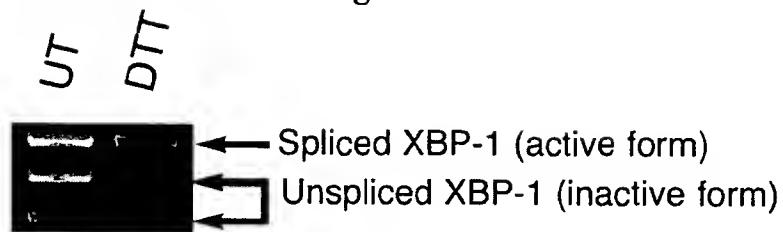


Figure 4

Figure 5
(1 of 2)

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DEFINITION Homo sapiens XBP1 mRNA for X box-binding protein spliced form, complete cds.
ACCESSION AB076384
VERSION AB076384.1 GI:18148381
KEYWORDS
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1
AUTHORS Yoshida,H., Matsui,T., Yamamoto,A., Okada,T. and Mori,K.
TITLE XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor
JOURNAL Cell (2001) In press
REFERENCE 2 (bases 1 to 1761)
AUTHORS Yoshida,H. and Mori,K.
TITLE Direct Submission
JOURNAL Submitted (18-DEC-2001) Hiderou Yoshida, Kyoto University, Graduate School of Biostudies; 46-29 Yoshida-Shimoadachi-machi, Sakyo-ku, Kyoto, Kyoto 606-8304, Japan
(E-mail:hid Yoshi@ip.media.kyoto-u.ac.jp,
URL:www.users.kudpc.kyoto-u.ac.jp/~p51907/mori/Index-mori.html,
Tel:81-75-753-4067, Fax:81-75-753-3718)
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Figure 5
(2 of 2)

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Figure 6
(1 of 2)

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DEFINITION Homo sapiens XBP1 mRNA for X box-binding protein unspliced form, complete cds.
ACCESSION AB076383
VERSION AB076383.1 GI:18148379
KEYWORDS .
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1
AUTHORS Yoshida, H., Matsui, T., Yamamoto, A., Okada, T. and Mori, K.
TITLE XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor
JOURNAL Cell (2001) In press
REFERENCE 2 (bases 1 to 1787)
AUTHORS Yoshida, H. and Mori, K.
TITLE Direct Submission
JOURNAL Submitted (18-DEC-2001) Hiderou Yoshida, Kyoto University, Graduate School of Biostudies; 46-29 Yoshida-Shimoadachi-machi, Sakyo-ku, Kyoto, Kyoto 606-8304, Japan
(E-mail: hid Yoshi@ip.media.kyoto-u.ac.jp,
URL: www.users.kudpc.kyoto-u.ac.jp/~p51907/mori/Index-mori.html,
Tel: 81-75-753-4067, Fax: 81-75-753-3718)
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**Figure 6
(2 of 2)**

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Figure 7

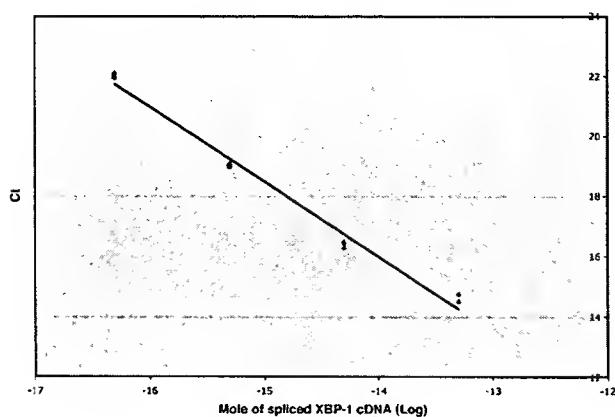


Figure 8

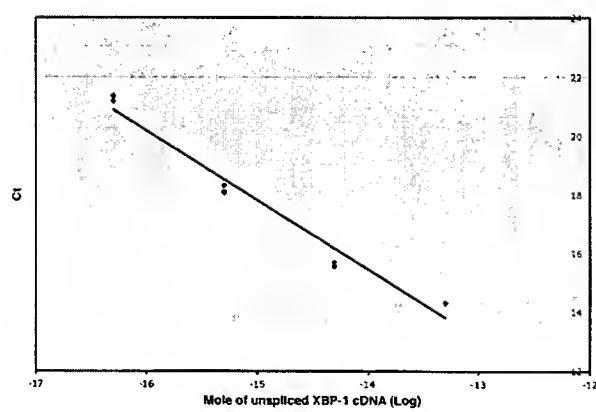
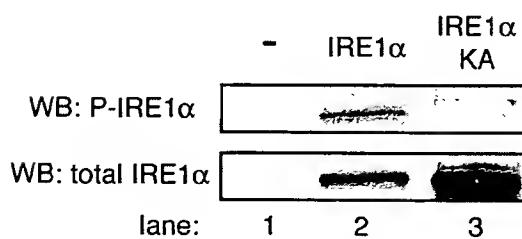


Figure 9



Figures 10A-10F

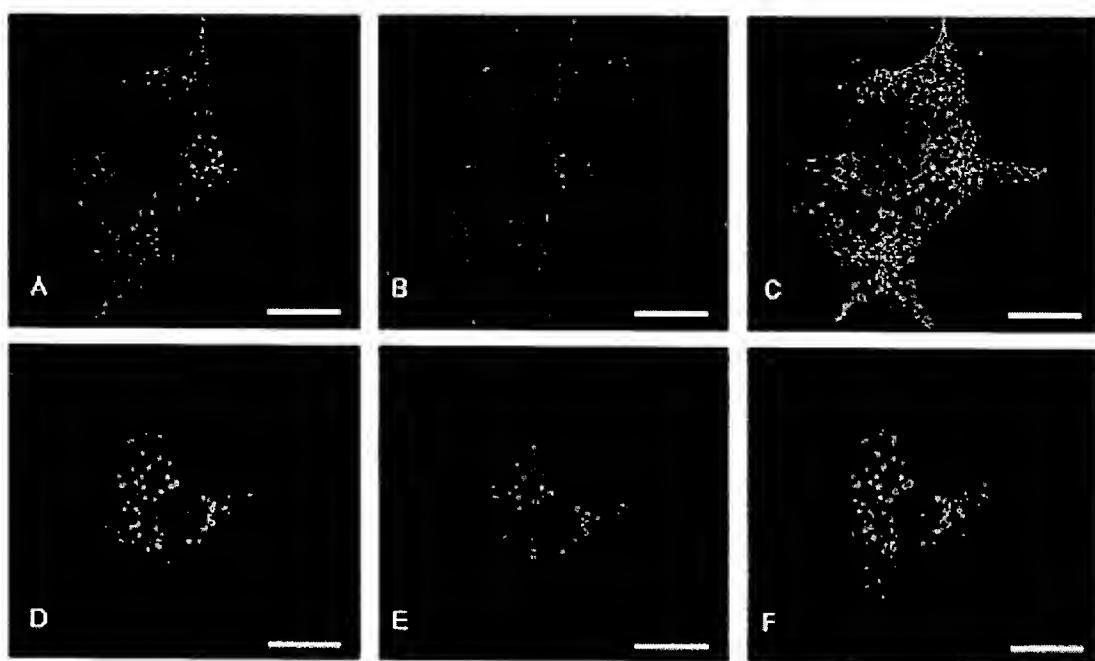


Figure 11A

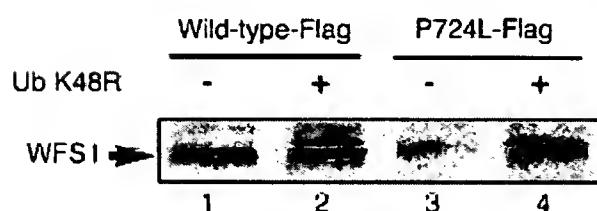


Figure 11B

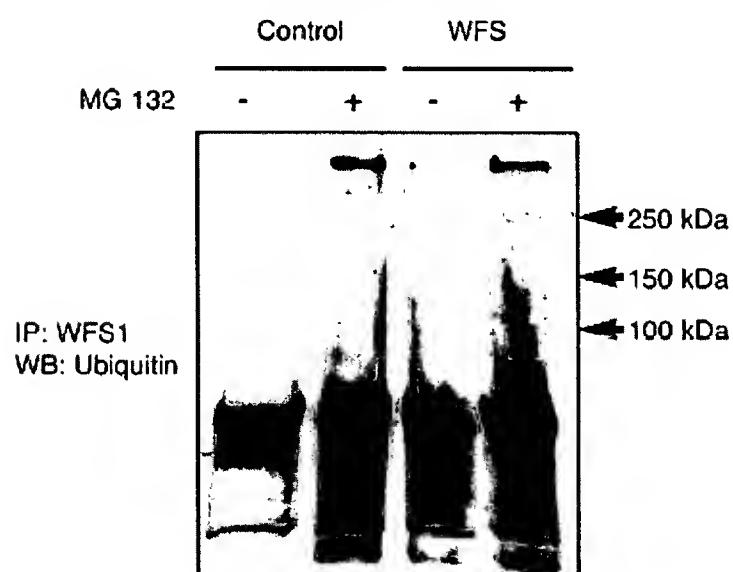


Figure 11C

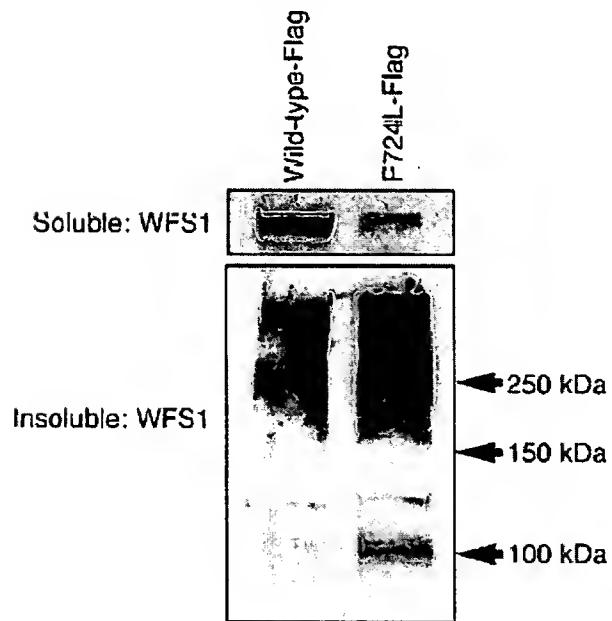


Figure 12A

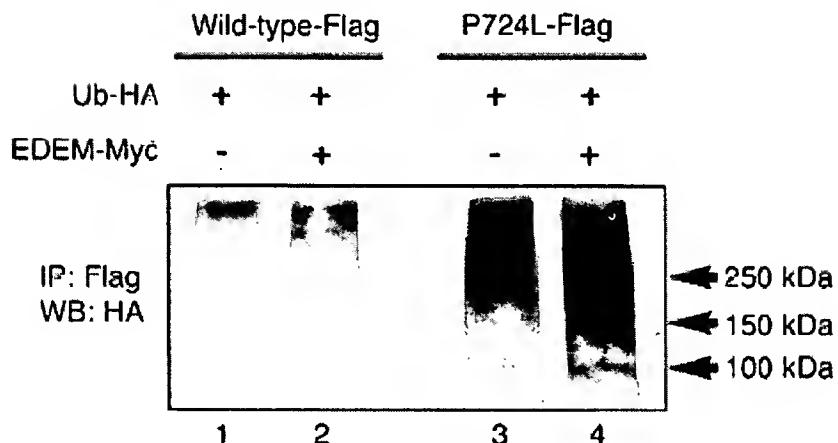


Figure 12B

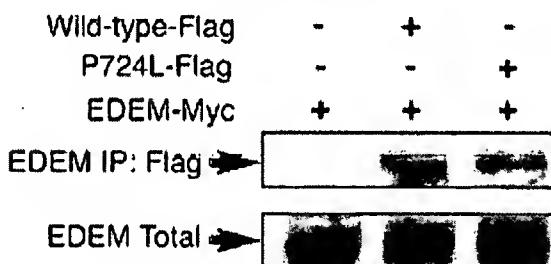


Figure 12C

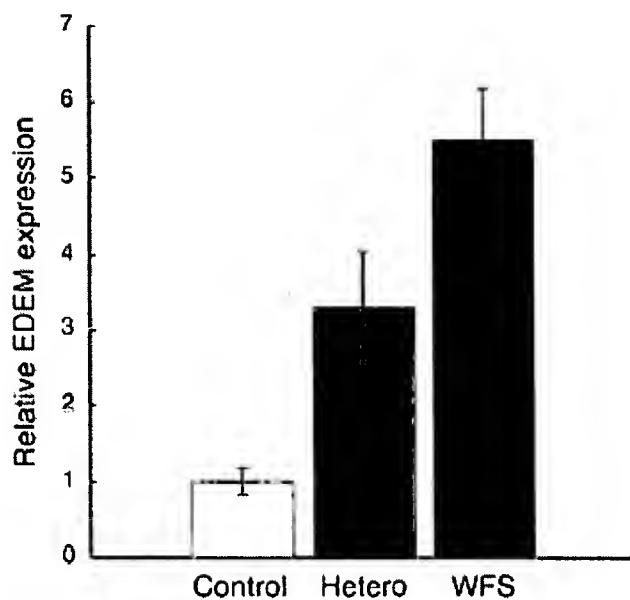


Figure 13A

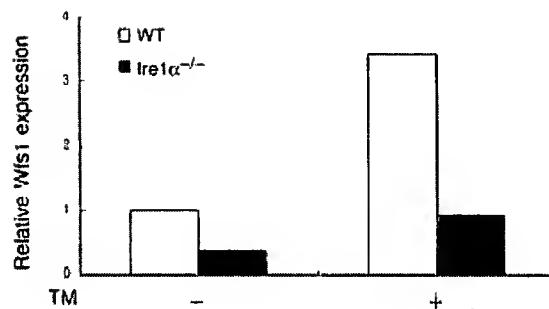


Figure 13B

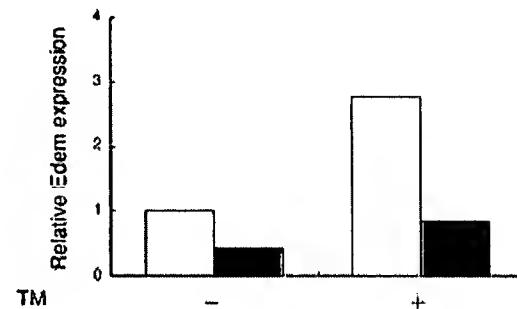


Figure 13C

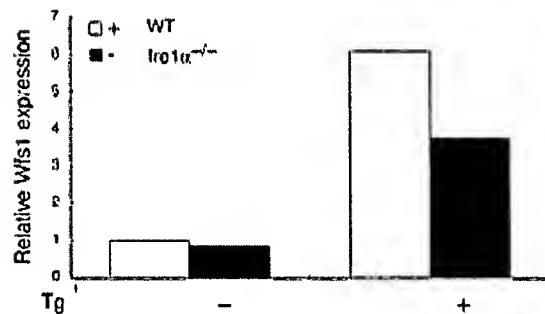


Figure 13D

